

EXPRESSION OF GLYCOPROTEIN gD AND EVALUATION OF IMMUNE RESPONSE OF BOVINE HERPES VIRUS TYPE-1 IN BUFFALO

Sumit Chowdhury^{*1}, Goutam Roy² and Bhaskar Sharma²

ABSTRACT: Bovine Herpes Virus type-1 (BoHV-1) causes a multitude of clinical symptoms in cattle, buffaloes and small ruminants. No effective live attenuated or killed vaccine is currently available and extensive research work in progress towards the development of the subunit and genetically engineered vaccine. Since DNA vaccine is currently regarded as most important breakthrough in vaccinology, the present work was aimed at construction of DNA vaccine using most immunogenic glycoprotein gD and studying its immune response and protection in buffalo. gD specific DIG labelled probe was used to screen gD specific clones from cDNA library. The gD specific cloned plasmid was purified for eukaryotic expression. The SDS-PAGE & Western blot analysis showed the transient expression of the expected 71 kDa gD following transfection in COS-7 cells. Four seronegative buffalo calves were immunized at 0, 30 and 60 days with recombinant purified plasmid and two calves were kept as control. The result of SNT, ELISA and MTT indicate gene specific seroconversion and CMI response following immunization with plasmid. At 86 days of post first vaccination, animals were challenged with virulent BoHV-1 (216/IBR). Hematological picture of the control animals showed leucopenia and that was due to destruction of lymphocytes shown by TLC and apoptosis study. Vaccinated animals showed reduced virus shedding in terms of days post challenge as well as titers compared to the controls. Based on the above findings, we concluded that DNA based vaccine induces specific and protective immune responses to the buffalo.

Key words: BoHV-1, DNA Vaccine, gD glycoprotein, pBK-CMV-NSJ.

INTRODUCTION

Bovine Herpes Virus type-1 (BoHV-1), major pathogen of cattle is associated with a variety of diseases like Infectious Bovine Rhinotrachitis (IBR), Infectious Bovine Vulvovaginitis (IPV) and mild conjunctivitis (Gibbs and Rweyemamu

1977). BoHV-1 is a member of alphaherpesvirinae sub-family (Roizman 1992). The virus is a great hindrance in artificial insemination programme (Thibier 1988). Like most herpes viruses, BoHV-1 remains latent after primary infection, a phase which is characterized

¹ Quality Control Officer, Frozen Semen & Bull Station, Composite State Animal Husbandry Farm, Salboni, Paschim Medinipur, West Bengal -721147, India.

² Division of Biochemistry, Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P- 243122, India.

^{*}Corresponding author

by lifelong persistence of viral DNA in trigeminal and in sensory ganglia (Ackermann *et al.* 1982). The genome of BoHV-1 is a linear of 136 kb with double stranded DNA with a total 73 open reading frame and ten genes coding glycoproteins mainly gK (UL53), gC (UL44), gB(UL27), gH(UL22), gM (UL10), gL(UL1), gG(UL4), gD(UL6), gI(US 7) and gE(US 8). Out of these glycoproteins, gD helps in penetration attachment and penetration and also highly immunogenic (Schwyzer and Ackermann 1996). The major glycoproteins gB, gC and gD, that are expressed at high level on the virion envelope and on the plasma membrane of the virus-infected cells, serve as immunogens. The sub unit vaccines against BoHV-1 utilizing these purified glycoproteins (gB, gC or gD) can induce protective immunity against BoHV-1 challenge (Babuik *et al.* 1987, Van Druen little van den Hurk *et al.* 1993). gD, a 71 kDa, glycoprotein is essential for virus replication and is involved in attachment penetration and cell fusion (Tikoo *et al.* 1995) and it induces a stronger and more consistent response than gB or gC (Hutching *et al.* 1990). The respiratory tract mucosal immunity directed against BoHV-1 gD forms an effective barrier against BoHV-1 replication and latency in cattle. The conventional vaccines *i.e.* live attenuated vaccine, killed vaccine and subunits vaccines do not provide solid protection as they render humoral response and there is short duration of immunity, requirement of sophisticated equipment leading to the high cost of production and problem associated to the handling of the virus in bulk. Both inactivated and modified live virus (MLV) vaccines against BoHV-1 are available. Although the currently available inactivated vaccines induce a good neutralizing antibody response, they are poor inducers of cell-mediated immune (CMI) response. Moreover, the antibody response induced by the inactivated vaccines is short lived.

Because the virus spreads via cell-to-cell transfer, and the infection proceeds in the presence of serum neutralizing antibodies, cytotoxic T-lymphocytes (CTLs) are critical for elimination of the virus (Tikko *et al.* 1995).

In recent years, DNA vaccine has emerged as one of the promising attractive vaccines for several viral diseases. Following vaccination DNA enters to the cells and expresses the desired protein which is then processed and presented to the class I MHC molecules which elicits a CD8+ cytotoxic T-cell response (McClements *et al.* 1996). Immunization with plasmid DNA elicits both humoral and cell mediated immune responses. The first attempts to make DNA vaccine in BoHV-1 was initiated by Cox *et al.*, 1993 by studying the immune response in mice and cattle with plasmid DNA coding gD of BoHV-1. Plasmid DNA coding glycoprotein gC confers partial protection in cattle against BoHV-1 when injected intramuscularly (Gupta *et al.* 2001). In India and elsewhere no reports of DNA vaccine for buffalo exist. Therefore present work was aimed to evaluate immune responses and protection label in buffalo injected with plasmid DNA vaccine of glycoprotein gD.

MATERIALS AND METHODS

Virus, vector and cell line

BoHV-1(216/IBRII) maintained in Immunochemistry Laboratory (ICL) in Biochemistry Division of Indian Veterinary Research Institute (I.V.R.I.), Bareilly, U.P was used as source of virus. Madin Darby Bovine Kidney (MDBK) cell lines in 60th passage level and COS-7 at its 7th passage maintained in I.C.L. were used for this study. cDNA library of BoHV-1(216/IBRII) prepared in pBK-CMV phagemid vector (stratagene, U.K.) was used as source for gD glycoprotein. XL0LR strain of *E. coli* was used for plating the excised phagemids.

gD specific probe preparation

MDBK cell line adopted purified BoHV-1 was the source of viral DNA which was extracted with proteinase K (250mg/ml) and with 5% SDS with incubation at 37°C for 15 mins and 60 mins respectively, followed by phenol-chloroform treatment. The purified viral DNA was used to amplify the gD using BG76F forward primer (5'-GGGCTGCAGC CCCGGCTGGG-3') and BG77R reverse primer (5'-GCAGGATCCGGGGCTAGGAGC-3'). Further amplified gene was confirmed by gD specific nested PCR primers, BG50F (5'-ATGAAGGGGAGACCGAGGAC-3') and BG51R (5'-GGCAGCGCGCTGTAGTTGAC-3'). The gD was labeled by BG76 and BG77 primers in PCR using digoxigenin (DIG labeling and detection kit, Boehringer, Mannheim) and purified by clean Genei kit (Bangalore Genei). The specificity of gD probe was tested with the BoHV-1 DNA with different dilutions (0.5 µg, 0.05 µg, 0.005 µg, 0.0005 µg & 0.00005 µg) using PCR product of M gene and F gene of Rinderpest virus and pBK-CMV plasmid vector as negative control.

Screening of cDNA library

Recombinant phagemid vector transformed in XL0LR cells of *E. coli* was plated on IPTG LB plate for blue white screening. Briefly, white colonies were picked up, grown in 200 µl of LB media having kanamycin (50µg/ml) in 96 wells plate, denatured with 25µl of denaturing solution (1 N NaOH, 3M NaCl, 0.2% SDS) for 5 mins & neutralized with 25µl of neutralization solution (1M Tris.Cl pH 7.5, 1.5 M NaCl). These samples were blotted on nylon membrane with bio-blot apparatus (Bio-rad). After drying, the membrane was baked at 120°C for 30 min, washed with 3X SSC (1% SDS) and hybridized with DIG labelled gD probe. All gD specific clones were picked up from 96 wells original plate, grown in LB-

kanamycin broth, plasmid was extracted and PCR was performed with external primers (BG76F & BG77R) & (BG50F & BG51R). Further, positive clones were double digested by EcoR-I & Xho-I at 37°C and result was analyzed in 1% agarose gel with ethidium bromide.

Sequence of the insert

The confirmed clones were further sequenced to present of the ATG in the insert. Briefly, 2µg of recombinant plasmid was denatured using 2 µl of 2 M NaOH in a total reaction of 40 µl. The mixture was then vortexed and incubated in room temperature for 10 min by adding 7µl of 3 M sodium acetate (pH 4.8) and 4µl of distilled water, precipitated by absolute alcohol, washed with 70% alcohol and dissolved in 10µl of distilled water, to that 2µl of annealing buffer, sequencing primers (10 pmol, 2µl), mixed & incubated at 65°C for 5 mins, transferred to 37°C water bath for 10 min incubation. In U shaped micro titer plate having A,T,G,C were mixed with radio-labeled ³⁵S dATP (BRIT, India) using T₇ DNA sequencing kit (Boehringer, Mannheim). The reaction mixture was loaded in sequencing SDS-PAGE gel containing 6% urea. After running, the gel was transferred to a membrane, dried up, loaded on a cassette with X ray film. It was kept at -20°C for exposure and developed after 2 days.

Eukaryotic expression

150 bp of the prokaryotic expression cassette was removed by Nhe-1 & Spe-1 using the Zap express Gold cloning kit (Stratagene, U.K.). The digested vector was self-ligated & transformed into the XL-1 Blue MRF' strain of *E. coli*, recombinant clones were further characterized by Pst-1 digestion and Sma-1 & Sal-1 digestion. Then the plasmid DNA was purified by midi prep endotoxin free kit (Qiagen) and the DNA concentration was checked in spectrophotometer.

5 µg of DNA was mixed with superfect (Qiagen) at a concentration of 3mg/ml and transfected in $4-5 \times 10^5$ cells of COS-7 cells in TC flask maintained in 5 ml DMEM with 10% BCS and incubated at 37°C for 48 hr. Cells were washed with PBS & lysed by 1X Laemmli sample buffer along with protease inhibitor and boiled for 10 min for analysis in SDS-PAGE and in Western Blotting.

Immunization of buffalo-calves

Two groups of buffalo consisting of Group-A of four animals numbered A1, A2, A4 & A5 and Group-B consisting of two animals as control were selected. Before DNA vaccination, blood was collected from each of the animals for serological screening. All animals of Group-A received 1 mg of DNA in 1 ml of NSS in two inoculated sites (left and right neck), whereas Group-B animal received nothing. At 30 days and 60 days of post immunization (d.p.i.), second and third injection was given with DNA at the same dose, site and route. All animals were challenged with 1 ml of 10^6 TCID₅₀/ml of freshly passaged BoHV-1 (216/IBR-II) by intranasal route and 0.5 ml intramuscularly.

Immune response in post challenged

Sera were collected from all the animals at 15 days intervals *i.e.* 15, 30, 45, 60 & 75 d.p.i. starting from first immunization and subjected to SNT & ELISA following standard protocol.

SNT

50µl of serial tenfold dilution of virus was made in micro titer plate (10^{-2} - 10^{-9}) in GMEM in duplication in each virus dilution. To this 50µl of constant dilution (1:8) of test sera (heat inactivated at 56°C for 45 min) was put. The virus serum mixture was then incubated at 37°C for 1 hr. 100µl of MDBK cells in GMEM at a cell concentration of 1.5×10^5 /ml were seeded to each

well and incubated. The serum neutralization index (SNI) was calculated by subtracting the SN titer from the virus titer.

ELISA

96 well ELISA plate was coated with 100µl of antigen (1:4000) diluted in carbonate-bicarbonate buffer, pH 9.6) in triplicate wells and incubated overnight at 4°C. Antigen used was the purified virus. The wells were washed thrice in PBS-T for 5 min each. Blocking was done with PBS-T+ 1% BSA (SRL-India) for 2 hrs at 37°C and washed as in previous step. The test sera diluted 1:4000 in PBS-T was added and incubated at 37°C for two hours followed by three washing. 50µl of Rabbit anti-bovine HRPO-IgG conjugate diluted 1:4000 in PBS-T was added and incubated for two hrs at 37°C. Followed by three wash as above, 50µl of substrate, O-phenylenediamine (Sigma) was added and kept in dark place for 15 min. Reaction was stopped and absorbance was taken at 492 nm.

MTT

Blood was collected in the presence of EDTA and peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (1.007, Sigma). The cells were adjusted to a concentration of 5×10^6 cells/ml in RPMI1640 media containing 10% fetal bovine serum (Hyclone). Subsequently, 100µl of cells were distributed into 96 wells microtitre plate. UV-irradiated killed BoHV-1 as antigen, conA (10µg/ml) and PHA (5µg/ml) as mitogens were used in 10µl volume in triplicate wells. After 72 hrs culture at 37°C in humidified CO₂ incubator, the proliferation response was measured by MTT dye assay (Bounous *et al.*, 1982) with slight modification. 20 µl of MTT (5mg/ml) was added to each well and plates were incubated further for 4 hrs at 37°C. At the end of incubation, 150µl DMSO was added to each well. After mixing the formazan by pipetting, the plates

were read at 570 nm with reference reduction at 650 nm. Proliferative responses were calculated as means of triplicate wells and expressed as stimulation index.

Immune response in post-challenge

Several parameters were chosen to evaluate the protective immunity for a period of 14 days post-challenged (d.p.c) with virulent virus. Thermal response (rectal temperature) was taken both in the morning & evening. Total leucocyte count (TLC) and differential leucocyte count (DLC) were done on 3, 5, 8 and 14 d.p.c. Virus isolation and quantification were taken by inserting the swabs through nostrils upto the turbinates and titrated at -2, 2, 4, 5, 6, 8, 12, 13 and 14 d.p.c. Apoptotic DNA fragments from the lymphocytes were performed as described by Duke & Cohen (1992).

RESULTS AND DISCUSSION

Preparation & characterization of gD specific probe

Genomic DNA of BoHV-1 was isolated from the purified virus by phenol: chloroform extraction. The concentration of the DNA was calculated from OD₂₆₀ as 1.3µg/ml & the OD_{260/280} was 1.764. The external primers consistently amplified 1406 bp of the gD gene, using the BoHV-1 DNA (Fig.1). In order to confirm the specificity, the 1405 bp PCR product was used to amplify which was given to a product size of 388 bp by internal primers (Fig.1). Double stranded DIG labeled DNA probe was generated by PCR amplification using external primers. The labeling & specificity was confirmed by hybridization. The genome of BoHV-1 DNA & PCR product of gD gave strong signal. No cross reactivity was demonstrated between the probe and PCR products of M gene & F gene of Rinderpest virus and pBK-CMV plasmid as such.

Screening of cDNA library

The cDNA library made in pBK-CMV phagemid vector (2AP express TM cDNA gigapack gold cloning kit, stratagene) was available in the laboratory. Plating of excised phagemid library and screening of recombinant clones were done by blue white screening, using XL0LR strain of *E. coli*. Out of 576 colonies, 7 hybridized specifically to the gD probe by presence of strong signal in the forms of dark spots. Further gD specific clones were confirmed by Kpn-I digestion to linearize a size of 7.5 kb, followed by EcoR-I & Xho-I digestion which gave 2.4 kb insert in five clones, other two clones having inserts of 3 kb & 1.4 kb respectively (Fig.2). All clones except one clone gave an amplification product of expected 388 bp when internal primers (BG50F & BG 51R) were used. The sequences of clones were corresponded with the known sequence of gD (Tikko *et al.*1995). Only one clone pBK-CMV-JIIF had the start codon, ATG in gD ORF (Fig.3).

Eukaryotic Expression

To remove the prokaryotic expression cassette, the plasmid was digested by Nhe-1 and Spe-1. The digested clones were purified and re-ligated by T₄ DNA ligase, transformed into XL-1 blue MRF' strain of *E. coli*. This plasmid designated as pBK-CMV-NSJ was extracted and digested with Pst-1 to confirm the removal of prokaryotic expression cassette as pBK-CMV-NSJ was undigested by Pst-1. Further confirmation of the clones were done by Sal-I and Sma-I digestion which generated three fragments of 4.6 kb, 1.7 kb and 600 bp and 5.1 kb, 1.375 kb and 600 bp respectively. Next the pBK-CMV-NSJ plasmid was purified using midi prep kit, concentration was 0.9µg/ml estimated by spectrophotometrically and existence of supercoil plasmid was confirmed in 1% agarose gel. 5µg of purified plasmid was used for transient

transfection in COS-7 cell line using 40µl of superfect reagent (Qiagen). After 48 hr incubation, cells were harvested and analyzed on SDS-PAGE which showed 71 kDa proteins (Fig.4). Expression of gD gene was further confirmed in western blot using BoHV-1 hyper-immune serum at a dilution of 1:100 and rabbit anti-bovine IgG-HRPO conjugate at a dilution of 1:1000. A band of expected molecular weight of 71 kDa was seen in the pBK-CMV-NSJ transfected cell lysate while the mock transfected cell lysate failed to show any band. Beside the expected molecular weight of gD, another band of 140 kDa was seen which confirmed the dimerization of gD protein (Fig.4).

Protection from challenge with BoHV-1

At 23 days past of second immunization, the animals were challenged with 1 ml of BoHV-1 virulent virus (216/IBR II) 10^6 TCID₅₀/ml by intranasal route and 0.5 ml of the same virus by intramuscular route. At the time of challenge, the vaccinated animals had significantly high antibody level than the unvaccinated control calves. After challenge, all immunized calves demonstrated a decline in ELISA titer and SNI at 3 d.p.c. At 14 d.p.c, the SNI of vaccinated animals was significantly higher than that of control animals. Like wise, the ELISA titer attained the pre challenge values at 14 d.p.c. and remained higher then the control animal . The control animals, did not demonstrate a rise in ELISA titer or SNI at 3 days, but a rise in these values at 11d.p.c.was recorded suggesting a primary immune response induced by the replication of challenged virus. The proliferative response of PBMCs at 3 d.p.c. was similar to pre challenge stature at 75 days past first immunization at 11d.p.c, the mitogen and antigen induced proliferative response was increased further.

After challenge with BoHV-1, the

unvaccinated control calves become sick with a sudden onset and recovered very slow. The animals of group A developed mild clinical symptoms. Accordingly, for an animal A1, a score of 1 was given by attending veterinarian at 2 and 3 d.p.c For animal A2, a score of 2 for 2 and d.p.c. and score of 1 for 4 to 11 d.p.c was assigned. For rest of the days, these animals were declared clinically healthy throughout the post-challenge observation period. For all immunized animals, the rectal temperature ranged from 101 to 102.2°F throughout the observation. However, subnormal temperature of 96.9°F to 99.8°F was recorded from 3 d.p.c to 5 d.p.c for the control animals. Thereafter, it increased but remained below 100.8°F till 8 d.p.c and subsequently attained normal values ranging between 101-102°F. Virus could be isolated at 3 and 5 d.p.c from three immunized calves in nasal swabs and at 6d.p.c. from the animal A2. These animals showed early clearance of virus. The titer ranged from 10^0 to 10^2 TCID₅₀/ml for these isolations. The isolated virus could be neutralized by BoHV-1 polyclonal serum. Subsequently virus could not be isolated from the immunized animals for next three consecutive days and further attempts for virus isolation ever not made. For unvaccinated animals, virus isolation was recorded upto 12 d.p.c in animal B-1 and 13 d.p.c in animal B-2 .

The TLC values revealed severe leucopenia in unvaccinated control calves. Three vaccinated calves had normal counts of total leukocytes at 3, 3 and 14 d.p.c However, animal A2 showed mode rate leucopenia at 8 d.p.c. The differential counts (DLC) indicated that leucopenia may be due to lower values of lymphocytes. At 5 and 8 d.p.c, we recorded an average of 27.42 and 22 ± 2.8 lymphocyte percentage from control group of animals. In vaccinated group DLC picture was largely normal. Apoptosis specific DNA laddering was observed in the lymphocyte chromosomal DNA when run on 1% agarose gel

8 d.p.c. for both animals of control group.

Buffalo plays a significant role in the dairy sector in India. BoHV-1 is endemic in this species of animals (Singh *et al.* 1986, Renukaradha 1993). Considering the limitations of conventional vaccines against BoHV-1 and the advent of DNA vaccines for infectious diseases, the gene for the full length of the gD of BoHV-1 under the control of cytomegalovirus (CMV) promoter was injected intradermally and its response was evaluated in buffalo calves in the present study. The envelop glycoproteins of BoHV-1 (gB, gC and gD) that are present on natural virus and on the virus infected cells, are major target in virus neutralization in infected animals and have been shown to elicit a cell mediated immune response (Denis *et al.* 1994). Immunization of cattle with a gD DNA vaccine induced an antibody response and rendered partial protection from the disease following challenged. (Cox *et al.* 1993). Another glycoprotein of BoHV-1 gB has also been included in the DNA vaccine formulation to induce immunity in cattle (Braun *et al.* 1998). Recently Gupta *et al.* (2001) evaluated that DNA encoding full length gC under RSV promoter induced gC specific protective immune response in cattle. Different studies have been reported to improve the gD DNA vaccine using native and secretory antigen (Lewis *et al.* 1997, Lewis *et al.* 1999, Van Drunen little van den Hurk *et al.* 1998). Any of three major glycoproteins (gB, gC or gD) individually or in combination induces protection from the disease caused by BoHV-1 infection in cattle (Babuik *et al.* 1999). Based upon the collective information, it is evident that gD is one of the most important protein (conclusive) of BoHV-1 and is the best candidate for development of new generation vaccines be it subunit, vectored or nucleic acid vaccines (Hutchings *et al.* 1990).

In the present study, intradermal route of

immunization, which is considered as one of the best methods by vaccine trials (Van drunen Little van den Hurk *et al.* 1993) was selected using syringe and needles being cheaper and convenient method. In buffaloes there is extensive connective tissue in the muscle, which may hinder DNA diffusion and transfection of the cells. The skin has specialized cells including langerhans cells, dendritic cells, keratinocytes and other immune cells giving relatively higher immunosurveillance compared to muscle (Bois and Kapsenber 1993). On intradermal immunization high transfection efficiency in these cells may result in better immune response (Progador *et al.* 1998).

A feasibly higher limit of 1 mg DNA per inoculation at two sites per animal was chosen and followed by a scheduled on 3 immunizations 30 days apart. We have selected a high dose and multiple inoculations as the process of DNA that enters, persists and functions in the injected tissue. There was weak immune response upto 30 days following first immunization as observed by Van drunen Little van den Hurk *et al.* (1998). The antibody response increased significantly at 60 days after second immunization. The third immunization did not elevate the SN index or ELISA titers further, indicating that the third vaccination is not necessary. gD vaccine also induced lymphoproliferative responses in calves. The immune response of gD vaccination here in buffalo is similar to the DNA vaccination studies reported earlier in the case of cattle (Cox *et al.* 1993, Van drunen little van den Hurk *et al.* 1998, Braun *et al.* 1998). Western blot analysis revealed the absence of gD-specific antibodies in pre-immunized serum. Presence of only gD specific antibodies in post-immunization serum indicates that the animals did not contact BoHV-1 during the experimental period. Our results demonstrate the ability of the injected plasmid containing gD gene of BoHV-1 to cause gene-specific sero

conversion in buffalo.

Based on earlier studies on cattle (Shukla 1987, Van drunen Little van dan Hurk *et al.* 1998) and in the absence of report on experimental infection of BoHV-1 in buffalo, prior to challenge, we evaluated clinical response of BoHV-1 (216/IBR II) in one buffalo calf by inoculating 10^6 TCID₅₀/ml virus. Taking clinical symptoms, thermal response and virus shedding into account, we calculated. The challenge dose as 10^6 TCID₅₀/ml by deep intranasal route, in order to test the efficacy of BoHV-1 gD DNA vaccine. The extent of anti BoHV-1 response rendered by gD vaccination was evaluated in terms of post-challenge antibody level against the BoHV-1 challenge. Third d.p.c, transient immuno-suppression was seen in all the vaccinated calves indicating an anamestic response against the virus, which was overcome there after. On the 14 d.p.c the level of neutralizing antibodies and IgG antibodies were increase to the extent of prechallenge values. This could be because of virus multiplication in the host which in the case of BoHV-1 induces immunosuppression. In control non-immunized calves, anti-BoHV-1 neutralizing antibodies were observed on 14 d.p.c suggesting a primary immune response induced by virus replication. It is plausible that the high levels of pre challenge anti gD antibodies served to partially prevent the BoHV-1 replication in immunized calves. Our results are expected in the light of the fact gD is found to induce a strong immune response and is the glycoprotein responsible for a significant reduction in viral replication and shedding (Hutchings *et al.* 1990). Monoclonal antibodies directed against this glycoprotein exhibited higher neutralizing titers than these of gB or gC, act at least partially by blocking virion penetration into the cells and function in the absence of complement (Dubussons *et al.* 1992). Accordingly, significant difference in the amount of virus shed post-

challenge was detected among the control and unvaccinated groups in the present study. Virus shedding from the nasal treat was detectable upto 5 d.p.c for all vaccinated animals (except animal A2 which shed BoHV-1 with a titer of 10^1 TCID₅₀ / ml at 6 d.p.c) as compared to 12-13 d.p.c in the case of control animals. Early clearance as well as significantly lower amount of shed virus in nasal swabs of vaccinated animals reflected the protective immune response of gD vaccination. The immunized calves (except A-2) did not developed clinical symptoms of the disease after challenge and all animals were recorded the rectal temperature value ranging between 101-102°F. The unvaccinated buffalo calves, when challenged, exhibited clinical symptoms typical of BoHV-1 except for pyrexia, subnormal temperature ranging between 96.9-100.8°F was observed (Gupta *et al.* 2001). No report of thermal response following challenge with BoHV-1 in buffalo is available. The reason for subnormal temperature could not be conclusively substantiated but it could be due to species variation in response to virus (or the characteristic of present virus isolate) and remain open for further investigation.

The gD specific immune response was found effective in preventing virus induced immuno-suppression in the immunized calves. The hematological picture of control unvaccinated animals post-challenge revealed severe leucopenia and DLC indicated that the leucopenia was due to destruction of lymphocytes. TLC and DLC counts of vaccinated animals were normal (barring A2 which showed a leucopenic TLC and lower percentage of lymphocytes in DLC at 8 d.p.c). The destruction of lymphocytes in control unvaccinated animals and the animal A2 of vaccinated group could be due to apoptosis. BoHV-1 is an immunosuppressive virus and is known to cause apoptosis (Hanon *et al.* 1996). In the present study apoptosis specific DNA

apoptosis in lymphocytes, possibly due to high levels of gD-specific antibodies in circulation in these animals. This finding corroborates the earlier report (Hanon *et al.* 1999) that monoclonal antibodies directed against gD but not gB or gC strongly reduced the high levels of apoptosis induced by BoHV-1. It is evident that gD is involved in the mechanism for induction of apoptosis by BoHV-1 and this evidence may be helpful in development of new vaccines or during pathogenesis of BoHV-1.

It is concluded that the early clearance of low titer virus and immunorestitution and protection from apoptosis following challenge with BoHV-1 that a DNA vaccine encoding gD induces protective immune response. It reduces the severity of infection, decreases virus transmission, thus reduces the economic loss due to diseased condition in dairy sector. It could be employed in multi-component vaccine formulation or along with respiratory tract mucosal immunity directed against gD to further improve upon the vaccine to curtail the establishment of latency.

REFERENCE

Ackermann M, Peterhans E and Wyler R.(1982). DNA of bovine herpes virus type 1 in the trigeminal ganglia of latently infected calves. *Am. J. Vet. Res.* 43: 36-40.

Babiuk LA, L'Italien JJ and van Drunen Littel-van den Hurk S.(1987). Protection of cattle from bovine herpesvirus type I (BHV-1) infection by immunization with individual viral glycoproteins. *Virology.* 159: 57-66.

Babiuk LA, Van Drunen Little-van den Hurk S and Babiuk SL.(1999). Immunization of animals: from DNA to the dinner plate. *Vet. Immunopath.* 72:189-202.

Bois JD and Kapsenberg ML.(1993). The

skin immune system: progress in cutaneous biology. *Immunol. Today.* 14: 75-78.

Braun RP, Babiuk LA, Loehr BI, Van Drunen Little-van den Hurk S.(1998). Particle-mediated DNA immunization of cattle confers long-lasting immunity against bovine herpesvirus-1. *Virology.* 265(1): 46-56.

Cox GJ, Zamb TJ and Babiuk LA.(1993). Bovine herpesvirus-1: immune responses in mice and cattle injected with plasmid DNA. *J. Virol.* 67: 5664-5667.

Denis M, Slaoui M and Keil G.(1994). Identification of different target glycoproteins for bovine herpesvirus type-1-specific cytotoxic T-lymphocytes depending on the method of in vitro stimulation. *Immunology.* 78: 7-13.

Dubuisson J, Israel BA and Letchworth III GJ.(1992). Mechanisms of bovine herpesvirus type1 neutralization by monoclonal antibodies to glycoproteins gI, gIII and gIV. *J. Gen. Virol.* 73: 2031-2039.

Gibbs EPJ and Rweyemamu MM.(1977). Bovine herpes viruses. Part I. Bovine herpesvirus-1. *Vet. Bull.* 47: 317-343.

Gupta PK, Saini M, Gupta LK, Rao VDP and Bandyopadhyay SK.(2001). Induction of immune responses in cattle with a DNA vaccine encoding glycoprotein C of bovine herpes virus-1. *Vet. Microbiol.* 78: 293-305.

Hutchings DL, van Drunen Littel-van den Hurk S and Babiuk LA.(1990). Lymphocyte proliferative responses to separated bovine herpesvirus-1 proteins in immune cattle. *J. Virol.* 64: 114-22.

Hanon E, Alian V, Lyaku J, Gunther K, Dennis M and Pastorel P.(1996). Inactivated Bovine Herpesvirus 1 Induces Apoptotic Cell

Death of Mitogen-Stimulated Bovine Peripheral Blood Mononuclear Cells. *J. virol.* 70:4116-4120.

Hanon E, Keil G, Sylvia van Drunen Little-van den Hurk Griebel P, Vanderplasschen A, Rijsewijk AM, Babuik L and Paul-Pierre Pastoret.(1999). Bovine Herpesvirus 1-Induced Apoptotic Cell Death: Role of Glycoprotein D. *Virology.* 257: 191-197.

Lewis PJ, Cox GJ, Van Drunen Little-van den Hurk S, Babiuk LA.(1997). Polynucleotide vaccines in animals: enhancing and modulating responses. *Vaccine.* 15(8): 861-864.

Lewis PJ, Van Drunen Little-van den Hurk S, Babiuk LA.(1999). Altering the cellular location of an antigen expressed by a DNA-based vaccine modulates the immune response. *J. Virol.* 73(12): 10214-10223.

McClements LW, Armstrong ME, Keys RD and Liu MA. (1996). Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of herpes simplex virus-2 disease. *Proc. Natl. Acad. Sci.* 93: 11414-11420.

Progador A, Irvine KR, Iwasaki A, Barber BH, Restifo NP, Germain RN.(1998). Predominant role for directly transfected dendritic cells in antigen presentation to CD8 T cells after gene gun immunization. *J. Exp. Med.* 188: 1075-1082.

Renukaradhya GJ.(1993). Sero-surveillance of cattle and buffaloes for infectious bovine rhinotracheitis infection by avidin-biotin ELISA. MVSc thesis. University of Agricultural Sciences. Bangalore.India.

Roizman B, Desrosiers RC, Fleckenstein B, Lopez C, Minson AC and Studdert MJ.(1992). The family herpesviridae: an update. *Archives Virology.* 123: 425-429.

Schwytzer M and Ackermann M.(1996). Molecular virology of ruminant herpesviruses. *Vet. Microbiol.* 53: 17-29.

Shukla DC. (1987). Studies on antigenic and immunogenic properties of infectious bovine rhinotracheitis virus. PhD thesis submitted to the Deemed University. Izatnagar. Bareilly. India.

Singh B K, Sreenivasan MA, Tongaonkar SS, Ramakant and Roychoudhary PN.(1986). Isolation of infectious bovine rhinotracheitis from semen and aborted materials of dairy cattle. *Indian J. Anim. Sci.* 56 (8): 823-826.

Thibier M.(1988). Artificial insemination and embryo transfer health control of breeding animals. *Bull. Elev. Fr. Suppl.* 22: 1-17.

Tikoo SK, Campos M and Babiuk LA.(1995). Bovine herpesvirus-1 (BoHV-1): biology, pathogenesis, and control. *Adv. Virus Res.* 45: 191-223.

Van Drunen Little van den Hurk S, Parker MD and Massie B.(1993). Protection of cattle from BoHV-1 infection by immunization with recombinant glycoprotein gIV. *Vaccine.* 11: 25-35.

Van Drunen Little van den Hurk S, Braun RP, Lewis PJ, Karvonen BC, Baca-Estrada ME, Snider M, McCartney D, Watts T and Babiuk LA.(1998). Intradermal immunization with a bovine herpesvirus-1 DNA vaccine induces protective immunity in cattle. *J. Gen. Virol.* 79: 831-839.